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# **IMMUNOREACTIVE PROTEINS IN *TAENIA SOLIUM***

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## ABSTRACT

*Taenia solium* cysticercosis is a neglected zoonotic disease that constitutes a serious public health problem in many low-income countries of Latin America, Africa, and Asia. Although diagnostic antigens are available, it is still necessary to identify new targets to improve the current diagnostic methods. Diagnostics tests are expensive, and most of them require the use of specialized equipment. Here, an expressed sequence tag (EST) library consisting of 5,760 sequences from *T. solium* cysticercus was constructed in which 1,650 unique sequences were identified, 845 of them previously unknown. Also, 2-DE was performed and succeeded in detecting several immunogenic proteins, among which TsolHSP36 and Tsol-p27 were identified by liquid chromatography-mass spectrometry (LC-MSMS), and Tsol-p27 was also found in our constructed EST library. A recombinant version of Tsol-p27 was produced and analysed by Western blotting, and it was recognized by sera from 13 NCC-positive humans but not by sera from control subjects. To develop a new and inexpensive diagnostic test that offers high sensitivity and specificity, we evaluated an immunodot blot assay using the previously analysed Tsol-p27 protein. The efficacy of the method was studied in comparison with the effectiveness of ELISA and of Western blot formats using the antigens TsolHSP36 and Tsol-p27. Compared to Western blot Tsol-p27, immunodot blot Tsol-p27 offered similar specificity (97.8% vs. 95.6%) but better sensitivity (86.7% vs. 76.4%). Also, sensitivity and specificity results were similar when comparing the ELISA and immunodot blot Tsol-p27 methods, and were lowest for Western blotting with TsolHSP36 (61.9% and 86.1%, respectively). Localization of Tsol-p27 was determined by immunohistochemistry using anti-rabbit Tsol-p27. Antibody response was observed in the parenchymal fold and tegument of the spiral canal. Sequencing analysis revealed that Tsol-p27 belongs to a group of proteins with the same bin/amphiphysin/rvs (BAR) domain as endophilin-B. This finding suggests that Tsol-p27 has functions related to membrane sorting, fusion, and transport, and that it may also aid growth of the parasite and support the adult tapeworm during colonization of the human intestine.

In conclusion, the present results show that the immunodot blot Tsol-p27 assay has good sensitivity and specificity, and it would be easy to implement this test in poorly equipped laboratories in endemic countries. Furthermore, this method is less expensive than ELISA and EITB analysis.

# LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. Lundström, J. **Salazar-Anton, F.** Sherwood, E, Andersson, B. and Lindh, J. Analyses of an expressed sequence tag library from *Taenia solium*, *Cysticercus*. *PLoS Negl Trop Dis*. 2010. 4 (12): p. e919
- II. **Salazar-Anton, F.** and Lindh, J. *Taenia solium*: A two-dimensional Western blotting method combined with the use of an EST-library for the identification of immunogenic proteins recognized by sera from neurocysticercosis patients. *Exp Parasitol*. 2011. 128 (4): p. 371-376
- III. **Salazar-Anton, F.** Tellez, A. and Lindh, J. Evaluation of an immunodot blot technique for the detection of antibodies against *Taenia solium* larval antigens. *Parasitol Res*. 2012. DOI: 10.1007/s00436-011-2747-z
- IV. **Salazar-Anton, F.** and. Lindh, J. Localization of Tsol-p27 and TsolHSP36, diagnostic proteins, in *Taenia solium* cysticercus. (Manuscript).

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## LIST OF ABBREVIATIONS

ATP	adenosine triphosphatase
BLAST	Basic Local Alignment Search Tool
CT	computed tomography
CSF	cerebrospinal fluid
CNS	central nervous system
cDNA	complementary DNA
CDC	Centers for Disease Control and Prevention
CE	Cystic echinococcosis
CSE	crude soluble extract
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EITB	enzyme-linked immunoelectrotransfer blot
ES	excretory/secretory
<i>E. coli</i>	<i>Escherichia coli</i>
FITC	fluorescein isothiocyanate
GST	glutathione S-transferase
HSP	heat shock protein
IPTG	isopropyl $\beta$ -thiogalactopyranoside
Ig	immunoglobulin
IF	immunofluorescence
ICP	intracranial pressure
KOBAS	KEGG Orthology Based Annotation System
KEGG	Kyoto Encyclopedia of Genes and Genomes
kDa	kilodalton
LAC	Latin American and Caribbean region
LLGPs	lentil lectin glycoproteins
LMM	low molecular mass
LC-MSMS	liquid chromatography-mass spectrometry
mAb	monoclonal antibody
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NTD	neglected tropical disease

NCC	neurocysticercosis
NCBI	National Center for Biotechnology Information
PHGPx	phospholipid hydroperoxide glutathione peroxidase
PF	parenchymal fold
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
<i>S. japonicum</i>	<i>Schistosoma japonicum</i>
TSC	tegument spiral canal
2-DE	two-dimensional electrophoresis



# 1 INTRODUCTION

## 1.1 GENERAL BACKGROUND

*Taenia solium* cysticercosis is a zoonotic disease that is often overlooked, even though it has serious public health consequences in many low-income countries in Latin America, Africa, and Asia. Indeed, cysticercosis is one of the NTDs, a group of chronic, debilitating, and poverty-promoting infections that are caused primarily by parasites, bacteria, and also some viruses and fungal species, and mainly affect the poorest people living in countries with low socioeconomic status [1].

The NTDs are known as infections of poverty because of their impact on child development, pregnancy outcomes, and worker productivity [1]. It has been estimated that 40% of the 556 million people living in the LAC region live below the poverty line, and 47 million of those individuals have to manage on less than 1 USD per day and another 74 million on less than 2 USD per day [2]. Cysticercosis is endemic in low-income countries, where transmission is associated with pigs raised in close contact with humans and poor hygiene and sanitary conditions.

The current methods used to diagnose human NCC include neuroimaging tests (i.e., MRI and CT), followed by a confirmatory serological method. Neuroimaging techniques are expensive and are seldom available to the majority of the population in endemic regions. Other diagnostic tools include histological identification of parasites in biopsies or CSF and immunological methods. Among the latter, ELISA and EITB assays are used most often to diagnose human cysticercosis [3], and both those techniques are specific and sensitive. However, they have the disadvantage of requiring expensive, specialized equipment that must be run by trained technicians, which limits the use of ELISA and EITB in poorly equipped laboratories in endemic countries. Thus there is a continuing need for alternative diagnostic methods that are practical, reproducible, and economical, and also reduce the amount of crude material required for analysis [4-6].

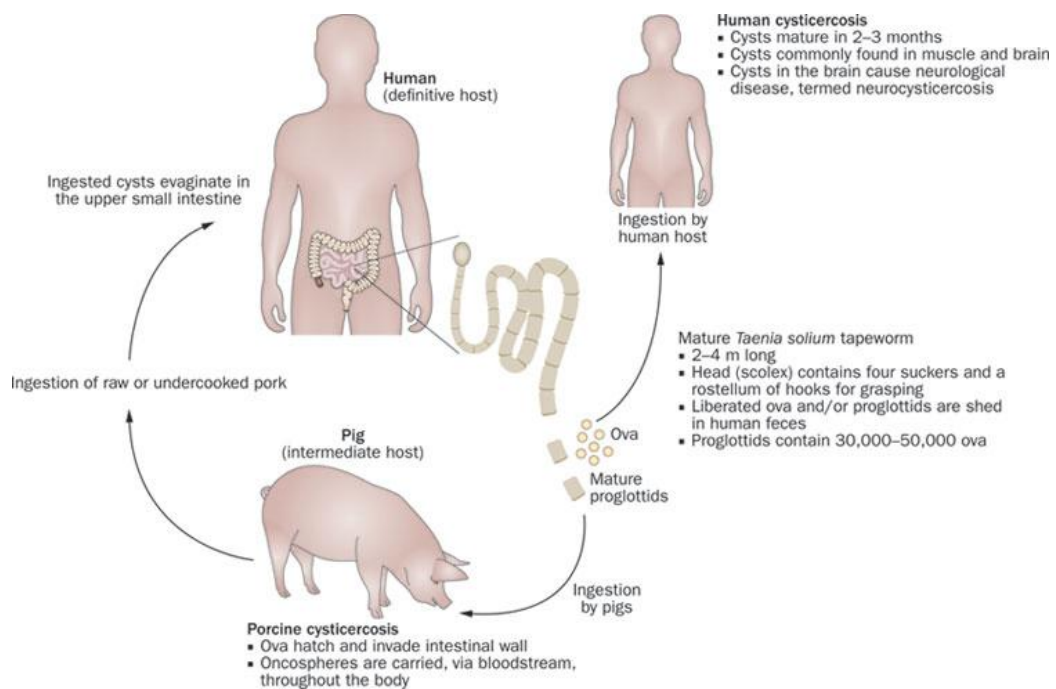
Cysticercosis is also an emerging disease in some high-income nations as the result of international travel and immigration from endemic areas [7]. Furthermore, infection with the pork tapeworm still represents a public health issue in endemic countries,

because thousands of infective *T. solium* eggs are released from humans and give rise to NCC, the main clinical form of cysticercosis [8]. This disease has been seriously neglected due to the lack of information and understanding of the problem in many countries.

## 2 BIOLOGY OF *TAENIA SOLIUM*

### 2.1 LIFE CYCLE

In the life cycle of *T. solium*, humans are the definitive host for the adult tapeworm, and pigs are the intermediate host for the cysticercus (Fig. 1). Humans harbour adult tapeworms, and eggs from the parasite are spread to the environment in faeces from the host. When a human ingests undercooked pork containing a cysticercus, the larva evaginates, attaches itself to the host's duodenal wall, and develops into an adult tapeworm. The adult worm attaches itself to the intestinal mucosa by means of a scolex equipped with four lateral suckers and a rostellum that bears 25–50 hooklets. Thereafter, the adult tapeworm grows and produces hundreds of hermaphroditic proglottids, which differentiate into gravid proglottids that are full of infective eggs. The eggs are spherical and measure 30–40 µm in diameter [9].



**Figure 1.** Life cycle of *T. solium* [8].

The fertilized eggs detach daily from the adult tapeworm and are expelled into the environment by passive discharge in the host's faeces. These eggs may in turn contaminate the soil and vegetables and other food prepared under deficient sanitary conditions. After being liberated from the proglottids, the eggs can be ingested by pigs and humans. Once they reach the digestive tract of the host, the eggs lose their coat through the digestive effects of the gastric and pancreatic enzymes, and they are

liberated as hexacanth embryos or oncospheres. Thereafter, the oncospheres cross the intestinal wall, enter the systemic circulation, and are transported to various organs in the host, including skeletal muscles, the CNS, and subcutaneous tissues. In those locations, the oncospheres acquire a vesicular shape and become cysticerci over a period of two months. The life cycle of the parasite is completed when undercooked pork containing cysticerci is ingested by a new human host [10-11].

Development of the larval stage of *T. solium* can also occur in the carrier of an adult tapeworm through what is called exogenous autoinfection. This involves ano-oral contamination and endogenous autoinfection in which eggs from the adult tapeworm living in the small intestine return to the stomach as the result of reverse peristalsis. Lack of hygiene and living with carriers of adult tapeworms are recognized as high risk factors for contracting cysticercosis [12-13].

## **2.2 TAENIA SOLIUM MORPHOLOGY**

**Adult tapeworm:** The adult tapeworm stage of *T. solium* is specific to the human host and has not been found in other species under natural conditions. The adult tapeworm is flat, cream coloured, and segmented, and it can be up to 8 m long and lives in the small intestine of the host [14]. Morphologically, the tapeworm is composed of a head or scolex that is 1 mm in diameter and equipped with four muscular suckers for attachment. The suckers contain an armed rostellum bearing a double row of 22 to 36 hooks. Behind the scolex is a thin, poorly differentiated neck that is approximately 5 to 10 mm long and consists of germinative tissue that shows biokinetic activity; it is from this part of the worm that the entire body or strobila is formed. The strobila is made up of 400–800 segments known as proglottids, which are covered by a tegument that forms the absorption surface through which the adult tapeworm ingests metabolites from the host [10, 15-16]. The strobila comprises immature, mature, and gravid proglottids, and those that are immature are undifferentiated and located closest to the neck. Mature proglottids contain male and female genitalia, and self- or cross-fertilization occurs after 2 or 3 months. Gravid proglottids are located at the tail end of the adult tapeworm, and each one contains a branched uterus packed with infected eggs that are embryos, also called oncospheres, and these are shed into the environment with the faeces of the adult tapeworm carrier [14, 17].

**Oncospheres:** An oncosphere in the adult tapeworm uterus is 29 to 77  $\mu\text{m}$  in diameter and consists of an embryo that is enclosed within a chorionic membrane, an embryophore, an embryophore membrane, and two oncosphere membranes. The oncosphere is also referred to as a hexacanth embryo, because it has three pairs of hooks [14].

**Cysticercus:** The larval stage or “*Cysticercus cellulosae*” is an ovoid translucent vesicle that is 0.5 to 2.0 cm in diameter. It is filled with a colourless liquid and contains an invaginated scolex (larval head) that is equipped with four suckers and an armed rostellum with two rows of hooks. The wall of the bladder is a membranous structure composed of three layers: a cuticle or external layer, a cellular or middle layer, and a reticular or internal layer [18]. The morphology of the cysticercus can vary and include an irregular form known as a “*racemose cysticercus*”, which looks like a large round or lobulated bladder, or a cluster of grapes. The racemose form lacks a scolex and can be up to 10–20 cm in size and contain several millilitres of vesicle fluid [19].

The cysticercus parenchyma consists of a complicated system of cells, slits, fibres, and channels that permits invagination of the scolex. The invaginated scolex forms a spiral canal, and the suckers are located at the end of the canal from which the armed rostellum arises. When a cysticercus is exposed to trypsin or bile *in vitro*, the larva evaginates in a process involving emergence of the scolex from the spiral canal. It is assumed that the evagination *in vivo* releases the scolex into the host's duodenum and promotes attachment to the intestinal wall of the definite host [15]. The surface of the larva is a syncytial tegument that is covered with digitiform projections called microtriches that allow the parasite to absorb nutrients from the interstitial tissue of the host. The cysticercus is an acoelomate and lacks a digestive system, and thus it must have an intact tegumentary surface in order to survive [15, 20].

### **3 CLINICAL IMPORTANCE OF TAENIASIS AND HUMAN CYSTICERCOSIS**

#### **3.1 TAENIASIS**

The condition called taeniasis arises when an adult tapeworm resides in the lumen of a human intestine, and it occurs only in human hosts. From the perspective of disease prevention, this is an important advantage, because the infected humans become the central target for any control/elimination action at a population level. Most adult tapeworm carriers are asymptomatic, although some present with minor symptoms of abdominal pain, distension, nausea, and diarrhoea, and some note the passage of proglottids in their faeces [21-22].

Two drugs are commercially available for the treatment of adult tapeworm infections: praziquantel and niclosamide. Niclosamide is the best choice, because it is not absorbed from the intestinal lumen [23].

#### **3.2 HUMAN CYSTICERCOSIS**

Cysticerci can become established in a wide of range of locations, including subcutaneous tissue, skeletal muscle, myocardium, the eyes, and the CNS. Subcutaneous cysticercosis entails the typical larvae in nodules that are painless for the host but surrounded by varying degrees of inflammation. Ocular cysticercosis can involve the subconjunctival space, extraocular muscles, aqueous or vitreous humour, the subretinal layer, or the optic nerve. In this disease, NCC is the most important clinical feature and occurs a larva is established in the brain. NCC in the CNS gives rise to symptoms through various mechanisms, such as local damage, inflammation, direct compression, blocking of CSF circulation, and vasculitis [23-24].

NCC entails infection of the brain by the larval stage of *T. solium*. NCC is the most common cause of acquired epilepsy in many low-income countries and accounts for over 20 million cases and 50 000 deaths each year. The clinical manifestations depend on the number, size, stage, and localization of the cysticerci. Seizures are the most common manifestation, occurring in 50% to 80% of patients [23, 25-26].

A cysticercus can reside in different intracranial and intracranial-spinal compartments. In the brain parenchyma, a cysticercus progresses through four phases called the vesicular, colloidal, granular-nodular, and calcified stages [27].

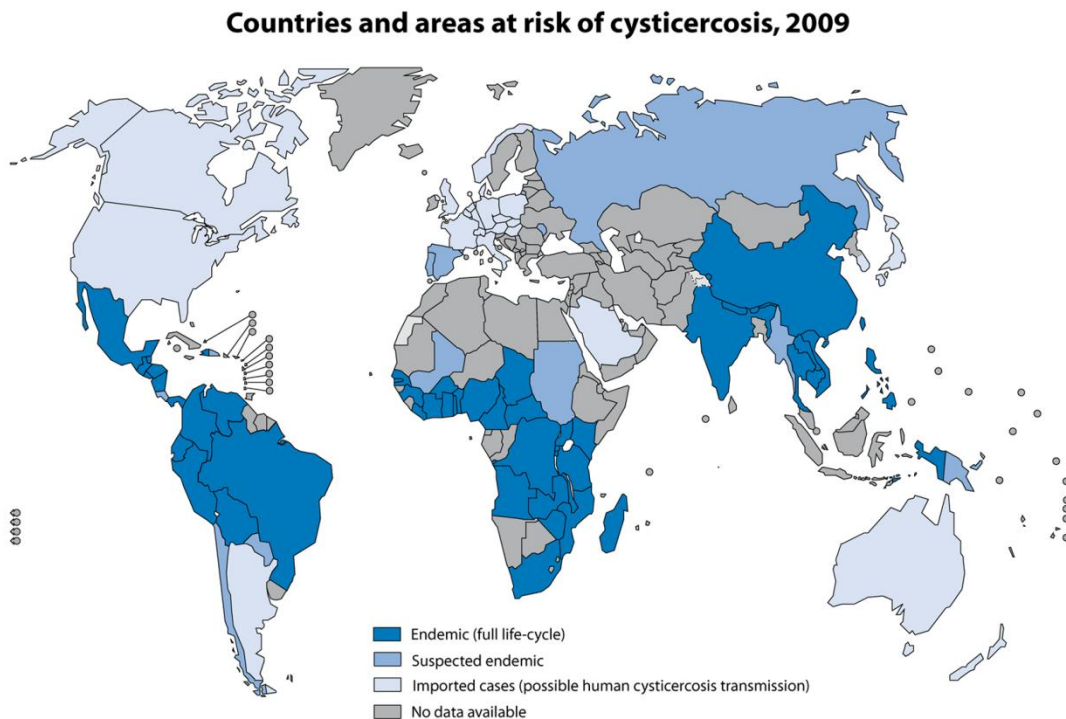
In the initial vesicular stage, the viable larva is surrounded by a clear fluid, and the affected human is asymptomatic. The colloidal stage arises during prolonged degeneration of the cysticercus, and it can induce an inflammatory reaction in the host. As the process of degeneration continues, the cysticercus starts to mineralize, most likely as the result of some degree of persistent inflammation, and thereby forms the granular-nodular stage. Finally, the granulation tissue is replaced by collagenous structures and calcification to produce the nodular calcified stage. All three of the degenerating stages can cause seizures in the host, and the colloidal stage can occasionally produce symptoms and signs of a mass lesion that raise the ICP. If there are numerous cysticerci in the brain, degeneration of the larvae can lead to severe oedema and markedly elevated ICP, which can cause headache, vomiting, altered sensorium, and death. This form of disease is termed cysticercotic encephalitis [25, 28].

Extraparenchymal NCC occurs when cysticerci enter the ventricular system where they cause hydrocephalus by blocking circulation of the CSF. The cysticerci can also settle in the subarachnoid spaces at the base of the brain or in the sylvian fissures, where they lose their scolices and creep along the subarachnoid spaces; this form of the parasite is called a racemose cysticercus. The symptoms associated with this condition are related to the severe inflammation of the vessels in the subarachnoid spaces, which is caused by the cysticerci and leads to infarction of the brain stem and the basal ganglia [29].

The treatment of NCC has advanced with the advent of the two drugs praziquantel and albendazole. Use of these agents should be individualized according to the immune response of the patient and the number, type, size, location, and stage of development of the cysticerci. Indiscriminate or unsupervised use can cause side effects, including dizziness, hepatic dysfunction, brain infarction, and death due to acute intracranial hypertension syndrome [30-31].

## 4 EPIDEMIOLOGY

Cysticercosis is a global disease that occurs primarily in low-income countries where pigs are raised and pork is consumed, and poor hygiene and sanitary conditions allow these animals to be exposed to human faeces. Epidemiological studies have demonstrated that cysticercosis is endemic in most countries in Latin America, Africa, and Asia (Fig. 2) [7, 32]. Latin American countries with prevalent cysticercosis include the following: Mexico [12], Guatemala [33], Honduras [34], Nicaragua [35-36], Bolivia [37], Peru [38], Ecuador [39], Colombia [40], Venezuela [41], and Brazil [42]. Cysticercosis is also an emerging disease in some high-income nations as the result of travel to or immigration from endemic areas [7].



**Figure 2.** Map showing the global distribution of taeniasis and *T. solium* cysticercosis in humans. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement. [[http://gamapserver.who.int/mapLibrary/Files/Maps/Global\\_cysticercosis\\_2009.png](http://gamapserver.who.int/mapLibrary/Files/Maps/Global_cysticercosis_2009.png); accessed January 2012].

It has been estimated that at least 50 million people in the world suffer from epilepsy, and more than 80% those individuals live in low-income countries. NCC is the most common cause of acquired epilepsy in many low-income nations and the most common



parasitic infection of the human nervous system, and it causes approximately 50,000 deaths each year [26, 43].

In Latin America, approximately 75 million people are at risk of NCC, and 400,000 are symptomatic [44]. The annual incidence of taeniasis has been reported to be from 10 to 50 million cases in humans and 50 million cases in pigs [45-46]. It has been proposed that *T. solium* cysticercosis should be declared an international reportable disease, which may not seem necessary considering that cysticercosis does not lead to sudden large-scale international outbreaks. However, *T. solium* cysticercosis belongs to the group of NTDs, and it is an indicator of poverty around the world [26, 47]. The social consequences of NCC are related chiefly to the seizures caused by the disease and include stigmatization and incapacitation leading to decreased productivity. In endemic countries, NCC affects older children and adults, which has a serious economic impact due to expected disability [48]. It has estimated that annual work productivity is reduced by up to 30% in people with NCC-induced epilepsy [49]. Porcine cysticercosis also has a significant economic impact, causing annual losses of 164 million USD in Latin America and approximately 25 million euros in ten Western and Central African countries [50-51].

## 5 DIAGNOSIS

### 5.1 TAENIASIS

Diagnosis of *T. solium* infection is based on a combination of comparative morphology, immunodiagnostic tests, and molecular methods. Macroscopic diagnosis of taeniasis is achieved through detection of *T. solium* eggs in human faeces, but this test offers only low specificity and sensitivity of 22.5% and 56%, respectively [52-53]. One disadvantage of this diagnostic procedure is that the eggs of *T. solium* and *T. saginata* are morphologically similar, and thus it is necessary to collect the adult tapeworm or proglottids to identify the species on the basis of morphological characteristics. *T. solium* is recognized by the presence of three ovarian lobes and the absence of a vaginal sphincter in mature proglottids, and the presence of 7–16 unilateral uterine branches in gravid proglottids [54-55].

The capacity to diagnose adult tapeworm carriers increased dramatically with the development of an ELISA test for *Taenia*-specific coproantigens in faecal samples [33, 52, 55]. A Western blot assay has been developed that uses coproantigens of adult *T. solium* to detect specific antibodies in serum, and this method has been found to provide 95% sensitivity and 100% specificity, levels that are much better than those achieved by microscopy and comparable to those of coproantigen ELISA [56-57].

Several specific molecular methods have been developed to detect DNA in human faeces for diagnosis of taeniasis. Molecular approaches include PCR, PCR combined with RFLP analysis, and multiplex-PCR [54, 58]. These techniques are highly sensitive and species-specific, thereby overcoming the limitations of other diagnostic tests. However, high cost and lack of technical capacity for handling these molecular methods limit their use in *T. solium*-endemic countries.

### 5.2 HUMAN CYSTICERCOSIS

It can be difficult to diagnose human cysticercosis and NCC due to the lack of specific signs and symptoms of these diseases. NCC is diagnosed chiefly by neuroimaging techniques such as CT or MRI and confirmed serology. Immunodiagnostic methods can be divided into two main groups: those that use an antigenic mixture or single

antigens to detect antibodies against them, and those that use specific antibodies to find specific antigens in the samples [59-61].

### **5.2.1 NEUROIMAGING METHODS**

CT and MRI are used to identify cysticerci in the brain not only to confirm the aetiology of the diseases, but also to provide information on the number, localization, size, and stage of the larvae. CT can detect most cases of NCC, with exception of small lesions in the ventricles or basal cisterns. MRI is considered to be more accurate than CT for assessing the intensity of the infection and the location and stage of a cysticercus [23, 62]. Neuroimaging findings can be classified as consistent with NCC, or they can establish either a probable or definitive diagnosis of the disease [63]. The clinical findings indicating NCC are roundish cysticerci that are 1–2 cm in diameter, have smooth walls, and contain an eccentric dot (scolex). The presence of multiple lesions compatible with NCC is useful in confirming the disease [8].

### **5.2.2 ANTIBODY-DETECTING METHODS**

Infection with *T. solium* results in a response involving production of specific antibodies, which can be detected in serum, CSF, saliva, and tears [4]. The following are examples of the antigens that have been used to diagnose human cysticercosis: LMM antigens [64], ES antigens [64-65], CSE [64, 66], total saline extract [67], LLGPs [68], vesicular fluid [66], membrane and scolex extracts [66], recombinant proteins [6, 35], and synthetic peptides [69-70].

Several techniques for detecting antibodies in humans have been described, including radioimmunoassay, haemagglutination, the complement fixation test, dipstick assays, latex agglutination, ELISA, Western blot analysis, and immunodot blot assay [3, 35, 60]. Detection of antibodies is confirmatory rather than decisive, and can indicate prior exposure to infection and not necessarily current active infection with viable cysticerci. EITB analysis and ELISA are the antibody detection tests most frequently used to diagnose cysticercosis [71-72]. EITB has been reported to have 98% sensitivity and 100% specificity, although the sensitivity drops considerably in cases involving single cysts in the brain. This technique requires expensive reagents and advanced equipment, as well as trained staff to perform the test [73]. The antibody-detecting ELISA provides sensitivity and specificity similar to EITB, and use of recombinant antigens in ELISA format has been found to give 90% sensitivity and 100% specificity [74]. A limitation

of serological studies based on antibody detection is that active disease can be overestimated due to occurrence of a transient antibody response, which can be the result of exposure to *T. solium* eggs that do not develop into a viable infection. The antibody reaction may persist long after the parasites have been eliminated by an immune mechanism or drug therapy [60, 75].

Another tool has emerged for diagnosing human cysticercosis, which is not considered to be an immunodiagnostic method. This technique is based on PCR amplification of *T. solium* DNA present in the CSF of NCC patients, and it has been reported to provide high sensitivity (96.7%) [76].

Inasmuch as the EITB method is expensive and requires skilled technicians to run it, various other techniques have been implemented to diagnose NCC. One of these is the immunodot blot assay, which is easier to perform and cheaper, and has been shown to provide levels of sensitivity and specificity similar to those obtained by ELISA [35, 77-78].

### **5.2.3 ANTIGEN-DETECTING METHODS**

Samples of serum or CSF are used most frequently to detect antibodies against parasite antigens, and several assays employing either polyclonal or monoclonal antibodies have been developed for that purpose. The immunoglobulin used most often is IgG, because it is the predominant antibody detected in NCC; IgA, IgE, and IgM can also be detected in NCC patients but are of little diagnostic value [59].

Antigen-detecting methods that use mAbs against the related parasite species *T. saginata* have been developed. Two such techniques are ELISAs based on the mAbs HP10 and B158/B60, which have been validated and used routinely to detect parasite antigen [3, 79-80]. These two tests can identify patients with a viable NCC infection, but they are not as sensitive as antibody assays based on Western blot analysis [80-83].

It may be more appropriate to use CSF samples to diagnose NCC, since the cysticerci are located in the brain in this disease. However, sampling of CSF is more invasive and entails greater risks compared to blood sampling. Results of antigen ELISA will become negative three months after a successful treatment [83-84]. Considering the drawbacks of antibody-detecting systems in the clinical setting, it may be advisable to

instead use antigen detection to confirm the presence of live parasites, which may aid decisions regarding the use of anti-parasite treatment [79].

## 6 PREVENTION AND CONTROL

*T. solium* has a high reproductive potential, and it is plausible that an adult tapeworm can expel over 100,000 infective eggs each day. Moreover, cysticercosis is related to poverty, and hence costs and locally available resources should be taken into consideration in all strategies aimed at controlling this disease [14, 55].

Cysticercosis is considered to be potentially eradicable, because it involves a single definitive host. The human carriers of adult tapeworms represent the only source of infection for pigs, and the pigs constitute the only viable intermediate host. In addition, there is no major role of a wild cycle in the transmission of *T. solium* [85].

The main obstacles to eradication of cysticercosis are the following: the need for simpler methods for diagnosing the disease in humans and pigs; the limited availability of drugs for treating taeniasis and porcine cysticercosis in endemic areas; a general lack of awareness of the presence and impact of the disease in the affected communities. There are also other factors that contribute to perpetuation of the disease, such as the fact that most endemic communities have deficient sewage systems and latrines, and inefficient inspection to detect infected pork in slaughterhouses [35, 55].

The following measures are among those used to control taeniasis/cysticercosis: detection and treatment of infected carriers of adult tapeworms [9]; mass treatment with niclosamide or praziquantel in the human population; health education; targeted or mass treatment of pigs with oxfendazole; ensuring that pigs do not come in contact with infected faeces [39, 55, 86-87].

Another important component in the control of taeniasis/cysticercosis is potential development of vaccines, which could be used in three different ways to directly or indirectly reduce human NCC: humans could be vaccinated to prevent infection with cysticerci or against adult tapeworms, or pigs could be immunized against *T. solium* cysticercosis. Development of a vaccine to protect humans from cysticercosis would be directly beneficial, but production costs in that context would be extremely high [88]. Also, prevention of porcine *T. solium* cysticercosis would remove the source of infections with adult tapeworm in humans and therefore lead indirectly to a reduction in

the incidence of human NCC. The vaccine antigens that have shown promise in experimental trials were derived from *T. solium* oncospheres [89-90]. Also, three host-protective recombinant antigens designated To16, To18, and To45W were discovered in *Taenia ovis* [91-92] and have been used to gain knowledge regarding the development of oncosphere antigen-based vaccines against other related parasites [93]. Later, three homologues of To16, To18, and To45W were identified and cloned from *T. solium* [94-96]. The antigens Tsol16, Tsol18, and Tsol45 have been effective in inducing high-level (> 97%) protection in pigs, although thus far Tsol18 has proven most effective [88]. It is possible that the recently discovered Tsol15, which is a homologue of To45W, will be added to this group of antigens and tested as a possible diagnostic target or candidate vaccine for human or porcine cysticercosis [5].

## 7 AIMS

The specific aims of the research underlying this thesis were as follows:

- To analyse an EST library from *T. solium* cysticercus.
- To identify immunoreactive proteins from *T. solium* cysticerci.
- To develop and validate an immunodiagnostic test for human cysticercosis
- To localize the diagnostic antigens Tsol-p27 and TsolHSP36 in *T. solium* cysticercal tissue.



## 8 MATERIALS AND METHODS

### 8.1 LABORATORY PROCEDURES

This section briefly describes the general study design, the collection of serum samples, and the experimental procedures performed in the present research. Detailed information about the materials and methods can be found in the corresponding papers.

#### 8.1.1 SERUM SAMPLE COLLECTION

**Paper II:** Human serum samples confirmed positive for NCC by computer tomography were used to identify immunoreactive proteins. Preliminary evaluation was performed on samples from two groups of subjects: a positive control group comprising four patients with NCC confirmed by CT and ELISA (Cypress) and nine patients with positive serology by ELISA (Cypress), and a healthy control group consisting of 13 apparently healthy humans.

**Paper III:** To study the efficacy of an immunodot blot test using the Tsol-p27 protein, a total of 100 human serum samples were collected from epileptic patients who attended the health centres of the Departments of Chinandega and Rosita in Nicaragua between March and August 2010. Sixty serum samples with positive (30 sera) or negative (30 sera) IF serology for Chagas disease were also included.

#### 8.1.2 BIOINFORMATIC AND MOLECULAR METHODS

**Transformation and excision (Paper I):** This study used a cDNA library, Uni-ZAP XR (Stratagene), constructed from polyA<sup>+</sup> selected mRNA from 1.75 g of cysticerci isolated from muscle of a naturally infected Peruvian pig (kindly provided by Kathy Hancock, [CDC], Atlanta, GA, USA) [97]. The library was transformed into the ampicillin-resistant vector phagemid pBluescript SK (+/-) and host bacteria (XL0LR) by applying a mass excision protocol as described by the manufacturer (ZAP Express cDNA Synthesis Kit and ZAP Express cDNA Gigapack III Gold Cloning Kit, Instruction Manual, Stratagene) [5].

**Preparation and sequencing (Paper I):** Positive, colourless bacterial colonies were selected and placed in 96-well microtitre plates and sequenced using a Megabace 1000 sequencing system (Amersham Bioscience) [5].

**Assembly, sequence analysis, and signal peptide prediction (Paper I):**

Computational analyses were performed using Phred for base calling and Phrap to assemble sequences. Cross-matching was done to achieve queries within the EST library, to cut out vector sequences, and to assemble contigs [98-99]. To compare the generated ESTs with databases on both a protein and a gene level, BLAST searches were conducted at the NCBI (Bethesda, MD, USA). To exclude irrelevant or low BLAST scores, a cut-off E-value of  $< 10^{-5}$  was used. Translation of the DNA sequences into three different frames was performed using Virtual Ribosome version 1.1 [100]. To detect signal peptides, the D-score in the implemented neural network was applied together with Hidden Markov models [5].

**Taxonomic classification (Paper I):** For all the unique sequences, the most probable putative protein of each contig and singlet was assigned to one of seven different hierarchic taxonomic categories. In short, based on the BLAST results, the organisms producing the putative proteins were assigned to the taxonomic group closest related to *T. solium*, and BLAST scores with E-values  $< 10^{-5}$  were classified as unknowns [5].

**Gene annotation (Paper I):** The software Blast2GO was used as described earlier [101]. Sequences in FASTA format were loaded into the program, and the default settings were used to assign GO terms. From a BLAST search, the annotation of the sequences was performed and pie charts were made using 2<sup>nd</sup> level GO terms based on biological processes, molecular functions, and cellular components [5]. InterProScan was also used in the Blast2GO software, and results merged with the GO terms, as described elsewhere [101]. In addition, analysis of metabolic pathways also performed using Blast2GO together with KOBAS [102-103] and the KEGG database [101].

**Cloning, expression, and purification of Tsol-p27 and TsolHSP36 (Paper II):** The selected cDNA used to produce the recombinant Tsol-p27 and TsolHSP36 proteins was amplified using specific primers. The PCR products of the Tsol-p27 and TsolHSP36 genes were subcloned in Bam HI/XhoI and Bam HI/Eco RI sites of the expression plasmid vector pGEX-4T-1 (Amersham Pharmacia) downstream of GST. **Papers II–IV:** Recombinant GST fusion proteins were expressed in *E. coli* BL21 (Invitrogen) after induction with 1 mM IPTG (Sigma). The soluble recombinant proteins were purified using magnetic beads coated with glutathione and PreScission Protease (GE Healthcare) according to the manufacturer's instructions. **Paper III:** In addition to the

purification step with glutathione-agarose beads, Tsol-p27 antigen was further purified using his-selected nickel magnetic beads (Sigma) [6, 35].

**Sequencing of immunoreactive proteins (Paper II):** Spots that were recognized by positive human sera in the 2-DE Western blotting and identified in the 2-DE gel stained with Coomassie Brilliant Blue (Bio Rad) were manually cut out and stored at  $-20^{\circ}\text{C}$  until used. Native proteins were trypsinized, and five to 10 fragments were identified by LC-MSMS [6].

**Isolation of cDNA encoding Tsol-p27 and TsolHSP36 (Paper II):** The amino acid sequences corresponding to Tsol-p27 were found by searching our previously constructed EST library for *T. solium* cysticercus [5], and the sequence corresponding to TsolHSP36 has been described elsewhere [104].

**DNA and amino acid sequencing analysis of Tsol-p27 (Paper II):** The ESTs were translated *in silico* using Virtual Ribosome version 1.1, and the resulting sequences served as an *in silico* library of translated ESTs. Amino acid sequences of peptides derived from Tsol-p27 were identified, and the corresponding ESTs were isolated and sequenced. DNA sequencing was done using a MegaBace 1000 system (Amersham Biosciences). **Papers II and IV:** The sequencing analysis of Tsol-p27 was performed at NCBI using a BLAST search option with a cut-off E-value of  $10^{-5}$  [6]. **Paper IV:** To be able to describe the family domain corresponding to Tsol-p27, a cut-off of  $10^{-9}$  was employed in the search option.

**2-DE Western blotting (Paper II):** Immunoreactive proteins in *T. solium* cysticerci were identified by 2-DE Western blotting [6]. Samples were analysed in triplicate to assess the reproducibility of the protein patterns. The gels were either stained with Coomassie Brilliant Blue (Bio Rad) or blotted onto nitrocellulose membranes (GE Healthcare).

### 8.1.3 IMMUNODIAGNOSTIC METHODS

**EITB (Paper III):** EITB was performed according to the procedure recommended by the manufacturer (LDBIO Diagnostic, Lyon, France)[35].

**Western blot analysis:** The proteins separated by 2-DE (**Paper II**), the recombinant Tsol-p27 and TsolHSP36 antigens (**Papers III and IV**), and the extracts of adult tapeworm and cysticerci (**Paper IV**) were analysed by Western blotting. The experimental procedures are described in the respective papers [6, 35].

**Antibody-detecting ELISA (Paper III):** A commercial ELISA (Cypress) was used according to the manufacturer's instructions [35].

**Immunodot blot analysis of Tsol-p27 (Paper III):** Immunodot blot analysis was carried out as described elsewhere [35].

#### **8.1.4 IMMUNOHISTOCHEMISTRY ANALYSIS**

**Production of rabbit polyclonal serum (Paper IV):** The recombinant Tsol-p27 and TsolHSP36 were used to raise polyclonal antibodies in rabbits. On days 0, 28, 56, and 84, two male rabbits per antigen received four immunizations with the recombinant proteins (250 µg/rabbit) emulsified in 250 µl of Freund's complete (first immunization) or incomplete (second to fourth immunization) adjuvant. Serum samples collected before immunizations were used as negative control. Serum was separated and stored at -20 °C until used.

**Localization of Tsol-p27 and TsolHSP36 in *T. solium* cysticercus (Paper IV):** The anti-rabbit Tsol-p27 and TsolHSP36 immune sera were also used to localize the Tsol-p27 and TsolHSP36 proteins in *T. solium* cysticercal tissue.

**Phylogenetic analysis (Paper IV):** Multiple sequence alignments of proteins of the endophilin-B family were performed according to the ClustalW method [105]. The protein sequences were obtained from GenBank. A phylogenetic tree was constructed with the aid of the software Lasergene 9 (DNASTAR Inc., Madison, WI, USA). Confidence values were assessed from 1,000 bootstrap replicates of the original sequences data.

## **8.2 ETHICAL APPROVALS**

**Papers II and III:** Collection and analysis of serum samples were approved by the Ethics Committee of the Faculty of Medical Science at National Autonomous University of Nicaragua, León, Nicaragua (Permission nos. 5, 47, and 73).

**Paper IV:** Animal experimentation was carried out in accordance with the requirements of Karolinska Institutet, Solna, Sweden (Permission no. N417/10).

## 9 RESULTS AND DISCUSSION

### 9.1 ANALYSES OF AN EXPRESSED SEQUENCE TAG LIBRARY (PAPER I).

An EST library is constructed to describe genes that are expressed during a specific stage of the life cycle of an organism. Briefly, mRNA from a particular species in a specific stage of development is isolated, transcribed into cDNA, and sequenced. EST analysis is a very cost-effective approach for discovering novel genes and identifying different groups of proteins (e.g., those with signal peptides) [106-107]. The *T. solium* library consists of 5760 ESTs with 5551 readable sequences. After conducting base call and excluding short sequences and vector sequences, 4674 high quality ESTs were saved (GenBank accession numbers GT889435-GT894161). Use of the Phred and Phrap methods also identified 1650 unique sequences (Table 1).

**Table 1.** Summary of *T. solium* ESTs

Description	Number	Percentage
Total number of sequenced clones	5760	
Total number of successful sequences	5551	96.4% <sup>a</sup>
Number of high quality sequences	4674	84.2% <sup>b</sup>
Unique sequences	1650	35.3% <sup>c</sup>
Number of contigs	434	
Number of clones included in contigs	3462	74.1% <sup>c</sup>
Average clones per contig	7.9	
Number of singletons	1212	25.9%

Percentages were calculated as the following: proportion of the total of number of ESTs;<sup>a</sup> proportion of successful sequences;<sup>b</sup> proportion of number of high quality sequences.<sup>c</sup>

Using a BLAST cut-off E-value of  $10^{-5}$ , we determined the expression patterns of the 25 most abundant ESTs. The four sequences expressed most often belong to conserved genes found in eukaryotes, which encode the antioxidant enzyme PHGPx, the structural proteins tubulin and actin, and a lysosomal enzyme identified as ATPase. This analysis also revealed high expression of genes that have been proposed to be expressed on the membranes of oncospheres and involved in establishment of this larval stage in humans.

These genes include those that encode Tso31d [108], T24 [97], and HP6/Tsol18 [89], of which the protein Tso118 is a promising candidate for development of a vaccine (Table 2) [109-110].

One of the highly expressed sequences showed homology to the 45W family of antigens, which are known to be expressed at the surface of the oncosphere [111]. The protein Tso115 (contig 492) was found to have an open reading frame corresponding to a protein with a predicted molecular weight of 15.3 kDa. The similarities between Tso115 and *T. ovis* 45W antigens was demonstrated using a BLAST research option with an E-value of  $< 10^{-6}$ . Other Tso1 proteins have been suggested to be valuable target molecules for diagnostic and immunization purposes, and Tso115 may well be regarded as a potential diagnostic antigen.

Among the predicted secreted proteins, we also found two different proteinase inhibitors. One of these was a serine proteinase inhibitor and the other a Kunitz type 8 proteinase inhibitor; both these proteins have been identified in the cestode *Echinococcus granulosus* and have been suggested to be involved in the parasite-host interface. Putative roles of these proteins are to participate in immunomodulation of the host or to function in and interfere with host physiological processes during the initial stage of infection [112-113].

The *T. solium* EST library can also be used to identify new drug targets to treat NCC patients. To enable suggestion of new drug targets, 60 different metabolic pathways have been annotated and mapped, and these include 6-phosphofructokinase, glutathione synthetase, and thioredoxin glutathione reductase [114].

Thus EST libraries can be used to make predictions and to identify genes that might be important for discovering novel drugs or developing new diagnostic tools. Nevertheless, the results presented in the literature indicate that the functions of the vast majority of ESTs identified to date are still unknown.

**Table 2.** Summary of the 25 most abundant ESTs and their putative identity.

Contig	Putative identity (BLASTX)	Length (nt)	No. of ESTs	Percentage of high quality sequences.	Identified in other <i>T. solium</i> EST libraries	Poly-A tail identified
<b>476</b>	gi 189235991 ref XP_972419.2 PREDI CTED: similar to DNA-J, putative	1012	17	0.36%	+	-
<b>477</b>	gi 188485737 gb ACD50951.1 Nc-DigChim-324430 [synthetic construct]	1120	20	0.43%	+	-
<b>478*</b>	gi 59709858 gb AAW88559.1 oncosphere protein Tso31d [ <i>Taenia solium</i> ]	1011	21	0.45%	+	+
<b>479</b>	gi 149364041 gb ABR24229.1 glyceraldehyde-3-phosphate dehydrogenase [ <i>Taenia solium</i> ]	1182	21	0.45%	+	+
<b>480</b>	Unknown 6	851	21	0.45%	+	-
<b>481</b>	gi 37778984 gb AAP20152.1 alpha-actin protein [ <i>Pagrus major</i> ]	931	22	0.47%	+	+
<b>482</b>	gi 221113094 ref XP_002155286.1 PRE DICTED: similar to Annexin-B12	1167	24	0.51%	+	+
<b>483</b>	gi 116687782 gb AAT74668.2 cysteine-rich secreted protein 2 precursor	878	24	0.51%	+	+
<b>484</b>	Unknown 5	667	25	0.53%	+	+
<b>485</b>	Unknown 4	1086	28	0.60%	+	-
<b>486</b>	Unknown 3	767	29	0.62%	+	+
<b>487</b>	Unknown 2	442	30	0.64%	+	+
<b>488</b>	gi 13539680 gb AAK29203.1 AF22590 5_1ribosomal protein S15a [ <i>Taenia solium</i> ]	982	34	0.75%	+	+
<b>489</b>	dbj AB086256.1  <i>Taenia solium</i> mitochondrial DNA	983	35	0.75%	+	+
<b>490</b>	Unknown 1	991	37	0.79%	+	+
<b>491*</b>	gi 158934366 emb CAO82075.1 HP6 protein [ <i>Taenia solium</i> ]	1111	39	0.83%	+	-
<b>492*</b>	gi 2114399 gb AAC47532.1 45W antigen ToW5/7 [ <i>Taenia ovis</i> ]/Tsol15	1088	40	0.86%	+	+
<b>493</b>	gi 56753429 gb AAW24918.1 SJCHGC 05540 protein [ <i>Schistosoma japonicum</i> ]	965	58	1.24%	+	-
<b>494</b>	gi 37786712 gb AAP47268.1  t24 [ <i>Taenia solium</i> ]	817	69	1.48%	+	-
<b>495</b>	gi 256050212 ref XP_002569521.1 hyp othetical protein [ <i>Schistosoma mansoni</i> ]	813	70	1.50%	+	+
<b>496</b>	dbj AB086256.1  <i>Taenia solium</i> mitochondrial DNA	1235	74	1.58%	+	-
<b>497*</b>	gb AAH30393.1  ATPase, H <sup>+</sup> transporting, lysosomal V0 subunit B	803	99	2.12%	+	-
<b>498</b>	dbj BAD88768.1  tubulin	1865	143	3.12%	+	+



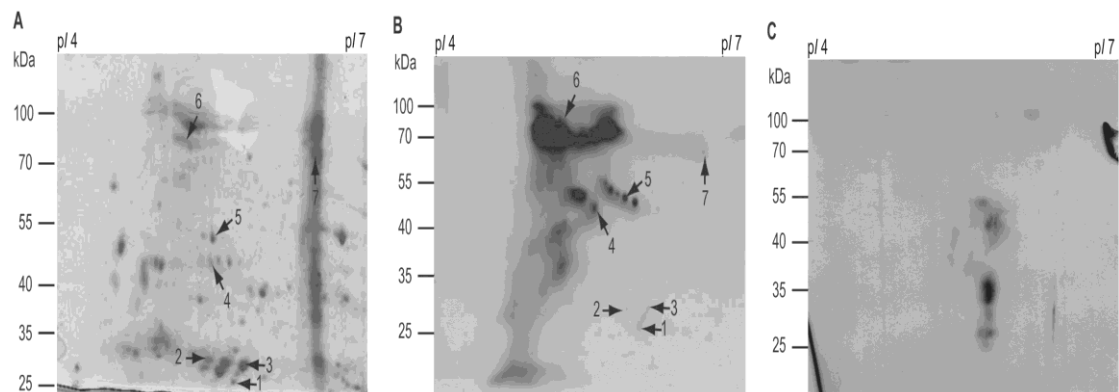
<b>499</b>	gi 207298859 gb ACI23578.1 beta-actin	1251	214	4.58%	+	+
<b>500</b>	gi 117956206 gb ABK58679.1 PHGPx isoform 1	1359	414	8.86%	+	+

BLASTX was used with an E-value cut-off of  $< 10^{-5}$ . \*Putative signal peptide.

## 9.2 IDENTIFICATION OF IMMUNOGENIC PROTEINS (PAPER II)

Identification of recombinant antigens for human cysticercosis can lead to easily obtained, low-cost substitutes for the native antigens, as well as a reproducible, field-applicable diagnostic assay [70]. At present, *T. solium* antigens for diagnosis of human cysticercosis are derived from parasites extracted from infected pork [4].

The main objective of the study reported in Paper II was to identify antigenic proteins and evaluate their potential in human NCC. Six immunoreactive proteins were identified by 2-DE Western blotting and sequenced by LC-MSMS. These antigens included phosphoenolpyruvate carboxykinase, 14-3-3 protein from *Echinococcus multilocularis*, actin, paramyosin, the small heat shock protein TsolHSP36 [104, 115-117], and the novel protein Tsol-p27 (Fig. 3).



**Figure 3.** Identification of immunoreactive proteins from *T. solium* cysticerci by 2-DE Western blot analysis. Total protein was extracted from cysticerci and loaded in the first dimension. Molecular size (kDa) is indicated to the left, and the designated proteins (arrows and numbers) were analysed by LC-MSMS. (A) A 2-DE gel stained with Coomassie Brilliant Blue. (B) A Western blot of sera from patients with NCC. (C) A Western blot of sera from a control group of NCC-negative individuals from the same area as the patients who provided the serum in panel B.

Tsol-p27 and TsolHSP36 were selected for recombinant production and preliminary serological evaluation. The other four immunoreactive proteins that were identified were not subjected to further study due to their conserved nature, and because they are present in other parasite-related species determined by a previous BLAST search

(NCBI). A cut-off E-value of  $1 \times 10^{-5}$  was used to exclude irrelevant or low BLAST scores.

Sequencing alignment of Tsol-p27 showed homology with the antigenic protein P-29 previously identified in *E. granulosus* [118]. P-29 is expressed in the larval stage of that species and is considered to be a suitable protein for immunodiagnosis of CE in humans [119].

To be able to compare the immunogenicity of Tsol-p27 with that of TsolHSP36 [104], it was also necessary to clone and express the latter protein. TsolHSP36 has previously been described as a target antigen in the diagnosis of human NCC [104].

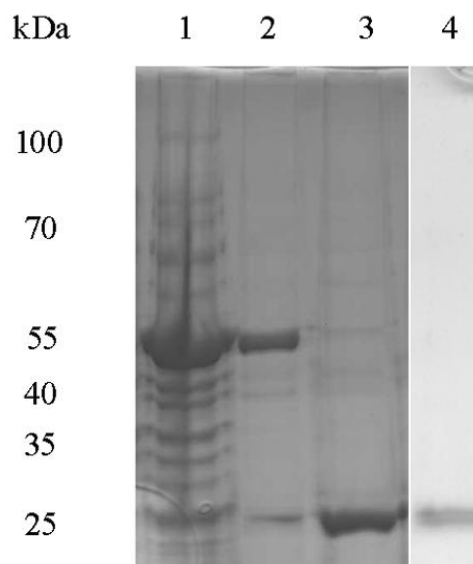
Western blot analysis of Tsol-p27 recombinant antigen was carried out using 13 serum samples from NCC-positive patients and 13 from NCC-negative subjects. TsolHSP36 was recognized by only two of the positive samples, whereas Tsol-p27 was recognized by all 13 positive samples. None of the proteins were recognized by sera from the NCC-negative group. Even though the number of serum samples evaluated in this study was limited, the results indicate that Tsol-p27 might be a suitable candidate for diagnosis of human NCC

### **9.3 EVALUATION OF AN IMMUNODOT BLOT ASSAY USING TSOL-P27 (PAPER III)**

The immunodiagnostic methods that are currently available for diagnosing human cysticercosis are specific and sensitive. However, they also have the disadvantage of requiring expensive and specialized equipment that must be run by specially trained technicians. Another potential drawback is that *T. solium* antigens for diagnosis of human cysticercosis are derived from parasites extracted from infected pork, which might decrease the sensitivity and specificity of the tests [4].

In this study (Paper II), Tsol-p27 antigen was identified in *T. solium* cysticercus extract by 2-DE Western blotting using human sera confirmed to be positive for NCC. To obtain a cheap diagnostic method that is easy to perform in poorly equipped laboratories, we developed and validated an immunodot blot assay using the recombinant Tsol-p27 antigen. To increase the efficacy and reduce the background, we purified the Tsol-p27 in two steps using glutathione-agarose and his-selected nickel

beads. The results obtained using our immunodot blot technique with the highly purified Tsol-p27 antigen showed a markedly decreased background compared to what could be achieved by Western blot analysis using one-step purified Tsol-p27 antigen. Figure 4 shows the Tsol-p27 antigen in SDS-PAGE, visualized by staining with Coomassie Brilliant Blue.



**Figure 4.** SDS-PAGE analysis of Tsol-p27 antigen. Lane 1, cells from induced *E. coli*; lane 2, purified recombinant GST/Tsol-p27; lane 3, Tsol-p27 digested with precision protease; lane 4, Tsol-p27 after purification using glutathione-agarose and his-selected nickel magnetic beads.

In order to evaluate the immunodot blot Tsol-p27 test, a commercial ELISA and the antigen TsolHSP36 and Tsol-p27 in Western blot formats were included in the serological evaluation. The EITB results were regarded as confirmatory of human cysticercosis and used as a standard to assess the performance of the diagnostic methods [71].

The sensitivity and specificity values were determined based on the positive and negative results obtained by EITB analysis. Of the 100 investigated serum samples from epileptic patients, 13 were positive and 87 negative by EITB. Table 3 summarizes the performance of the different immunodiagnostic tests in comparison with EITB as the gold standard method for detection of antibodies in serum from epileptic patients.

The immunodot blot Tsol-p27 test was comparable to the Western blot Tsol-p27 assay with respect to specificity (97.8% versus 95.6%) but not sensitivity (86.7 versus

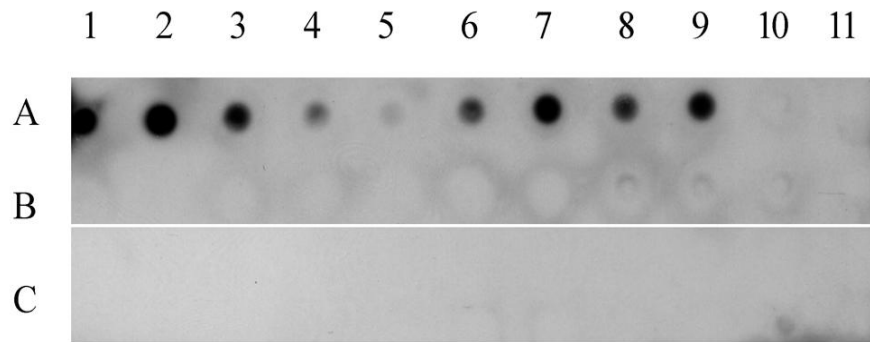
76.4%), and it was similar to ELISA regarding both specificity (97.8% versus 94.6%) and sensitivity (86.7 versus 86.7). Western blot TsolHSP36 showed lower specificity (86.1%) and sensitivity (61.9%) compared to the other immunodiagnostic methods. An example of the results obtained with the immunodot blot assay using the Tsol-p27 antigen is presented in Figure 5.

**Table 3.** Comparison of different immunodiagnostic tests with the EITB method as the gold standard for detection of antibodies against *T. solium* cysticerci in serum samples from epileptic patients

	<b>EITB</b>		
	Positive	Negative	Total
<b>Immunodot blot Tsol-p27</b>			
Positive	13	5	18
Negative	2	87	89
Total	15	92	102
<b>Commercial ELISA</b>			
Positive	13	5	18
Negative	2	87	89
Total	15	92	102
<b>Western blot Tsol-p27</b>			
Positive	13	4	17
Negative	1	87	88
Total	14	91	105
<b>Western blot TsolHSP36</b>			
Positive	13	14	27
Negative	8	87	95
Total	21	101	122

It has been reported that using ELISA to detect specific antibodies against *T. solium* cysticerci in samples from patients with suspected cysticercosis provides a useful tool for diagnosis of this disease [120]. However, the results of ELISA employed for this purpose vary significantly [36, 121], and the discrepancy between studies can be explained by the use of disparate criteria for patient selection and different methods as the gold standard.

The immunodot blot Tsol-p27 test showed good sensitivity and specificity in our study, and it is simpler to perform and less expensive than ELISA, EITB, and Western blot analysis. In addition, the immunodot blot method can be easily implemented in poorly equipped laboratories in endemic countries.



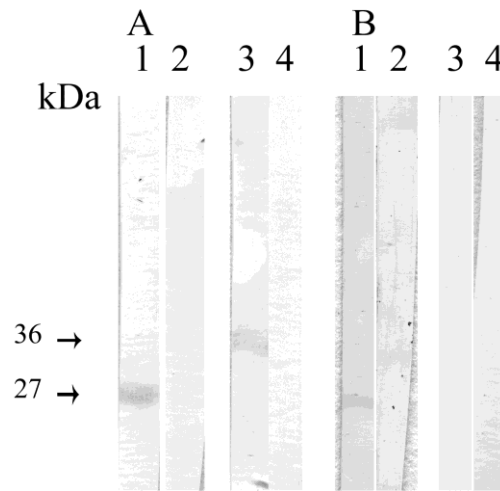
**Figure 5.** Immunodot blot analysis using the Tsol-p27 antigen. (A) Probing done with sera from two NCC -positive controls (1 and 2), seven positive epileptic cases (3–9), and two negative controls (10 and 11). (B) Probing done with negative serum samples from epileptic cases. (C) Probing done with sera from patients positive (1–6) or negative (7–11) for Chagas disease.

#### 9.4 LOCALIZATION OF THE PROTEINS TSOL-P27 AND TSOLHSP36 IN *T. SOLIUM* CYSTICERCUS (PAPER IV)

Recombinant antigens have been tested because they show potential for diagnosing human cysticercosis, and the recombinant proteins Tsol-p27 and TsolHSP36 show considerable promise in that context [6, 104]. Nevertheless, no data have been published regarding the localization of Tsol-p27 and TsolHSP36 in the *T. solium* cysticercus or the biological function of Tsol-p27. In our study (Paper IV), antibodies against Tsol-p27 and TsolHSP36 were produced in rabbits and used to analyse localization of these proteins in *T. solium*.

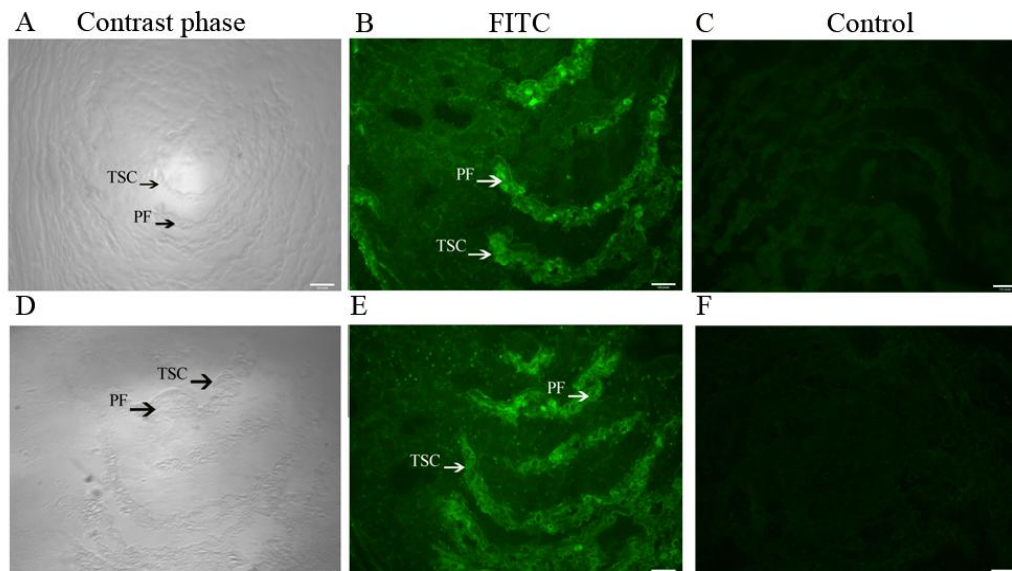
Antisera were found to contain antibodies specific to their respective antigens. In Western blot analysis, Tsol-p27 was detected in both the cysticercal and the adult stage, revealed as a single 27-kDa band, whereas TsolHSP36 protein was detected only in the cysticercus as a single 36-kDa band (Fig. 6).

Immunohistochemistry analysis revealed that Tsol-p27 and TsolHSP36 were reactive in the spiral canal and tegument of the *T. solium* cysticercus (Fig. 7). The presence of Tsol-p27 in the spiral canal may be related to growth and support of an adult tapeworm in the human intestine. Furthermore, Tsol-p27 may contribute to attachment of the scolex to the host's intestinal wall, where it must remain despite the strong peristaltic action of the intestine. The reaction of the parasite tegument to Tsol-p27 suggests that this protein is involved in the ability of the worm to absorb nutrients from the host [122-123].



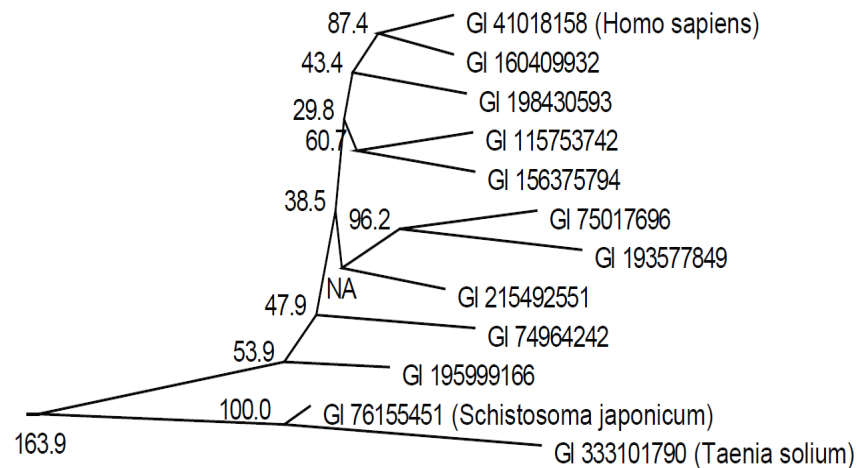
**Figure 6.** Western blot analysis of extracts of cysticercal (A) and adult (B) *T. solium* tissue using anti-rabbit Tsol-p27, TsolHSP36, and pre-immune sera. Lane 1 was probed with anti-rabbit Tsol-p27 serum, and lane 2 with pre-immune serum, lane 3 with anti-rabbit TsolHSP36 serum, and lane 4 with pre-immune serum.

Sequencing analysis of Tsol-p27 using a cut-off value of  $< 10^{-9}$  revealed an endophilin-B BAR domain. Members of this group of proteins are involved in clathrin-mediated endocytosis, binding to membrane vesicles, and induction of tubular conformation. These biological processes are associated with the endoplasmic reticulum, Golgi apparatus, and endocytic vesicles, where they are important for a variety of processes that require membrane sorting [124-125].



**Figure 7.** Sections of *T. solium* cysticerci photographed under phase contrast and an FITC filter. Panels A and D illustrate (arrows) the parenchymal folds (PF) and tegument of the spiral canal (TSC); panels B and E are images obtained using rabbit anti-Tsol-p27 and anti-TsolHSP36 proteins, with structures indicated as in A and D; panels C and F show results obtained with the pre-immune sera used as controls. Scale bars: 100  $\mu$ m in panel A and 200  $\mu$ m in panels B, C, D, E and F.

A phylogenetic tree was constructed to determine relationships between BAR regions of endophilin-B proteins from different species (Fig. 8). This analysis indicated that Tsol-p27 from *T. solium* was more closely related to the BAR region from *S. japonicum* than to other BAR regions within the group. Both these parasitic species belong to the phylum Platyhelminthes or flatworms.



**Figure 8.** Phylogenetic tree based on sequences of the endophilin-B protein. The multiple sequence alignments were obtained using ClustalW, and the tree was constructed with the software Lasergene 9 (DNASTAR. Inc., WI., USA). Bootstrap values from 1,000 replicates were used to assess the robustness of the generated tree.

The responses involving HSPs represent a general homeostatic mechanism that protects cells and organisms from damage caused by environmental stress [126-127]. TsolHSP36 is present in the *T. solium* cysticercus, where it is localized to the spiral canal and tegument. It is possible that a cysticercus can be exposed to strong variation in temperature while in the intermediate host (i.e., before reaching the final host), and it is also plausible that the host immune response can play an important role in the changes in temperature [126]. The presence of TsolHSP36 in the larval stage suggests that it is involved in promoting survival of the parasite by protecting it from the harsh environment.

## 10 CONCLUSIONS

**Paper I:** More than half of the sequences analysed in the EST library were of unknown function, and 845 novel sequences were identified. EST analysis is a very cost-effective method for discovering new genes and identifying different groups of proteins.

**Paper II:** Tsolp-27 and TsolHSP36 were identified and serologically evaluated. Results of Western blot analysis indicated that Tsol-p27 is a better antigen than TsolHSP36, and thus Tsol-p27 might be tested and evaluated as a suitable candidate for diagnosing human NCC.

**Paper III:** The immunodot blot Tsol-p27 assay performed well compared to other diagnostic tests. This method is simpler to use and less expensive than ELISA, EITB, and Western blot analysis. It would be easy to implement the immunodot blot Tsol-p27 test in poorly equipped laboratories in low-income countries.

**Paper IV:** The function of Tsol-p27 might be related to a variety of biological processes that involve membrane sorting, fusion, and transport, and it might also aid and promote the growth of the adult tapeworm within the human intestine.



## 11 ACKNOWLEDGEMENTS

The research findings presented in this thesis are the result of teamwork, and it would not have been possible to finish this project without the support and help of many people. In particular, I would like to express my gratitude to the following individuals:

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